

REMARKS

In the Claims

Applicant respectfully requests entry of new claim 64. Support for new claim 64 can be found in the original claims and through the specification, for e.g., support for the sources of the RNA can be found at:

page 4, line 18:

The naturally occurring RNA can be derived from a biological material such as bacteria, viruses such as those causing infection in humans, animals or plants, viroids, or cells such as fungal, animal and plant cells.

An important aspect of this invention is modification of mRNA, rRNA and viral RNA since they are of major scientific and clinical interest and serve as a good example of the problems encountered when manipulating RNA. The invention further provides methods for obtaining intact full-length copies of mRNA, rRNA, viral RNA and other types of RNA isolated from cellular sources or extracellular fluids that demonstrate increased stability in conditions that would otherwise destroy a major fraction of the unmodified RNA.

and page 23, line 19:

... This process may be automated in order to detect for example RNA viruses such as HCV and HIV in blood or body fluids. The immobilised sample might then be used for any number of downstream applications such as hybridisation, RT-PCR, TMA or NASBA.

Applicant notes the claims 1-7 were incorrectly numbered in the preliminary amendment. Claims 1-7 should have been number 57-63 respectively. The proper numbering is indicated in the claims listed.

Arrangement of the Specification

In response to the Examiner objection to the arrangement of the specification, Applicant provides herewith a marked-up substitute specification amended to include the headings listed in the office action. No new matter has been added. Accordingly, Applicant respectfully requests reconsideration and withdrawal of the objection.

Informalities of the Specification

In response to the Examiner objection the status of the patent applications listed in the specification have been updated and now include publication dates and numbers. Further, the specification has been amended to include a "Brief Description of the Drawings" heading above

the descriptions of Figures 1 and 2. Applicant further corrected misspelled words and incorrect punctuation as indicated. Applicant has corrected the specification so that trademarks are now in all caps. No new matter has been added. Accordingly, Applicant respectfully request reconsideration and withdrawal of the informalities of the specification objection.

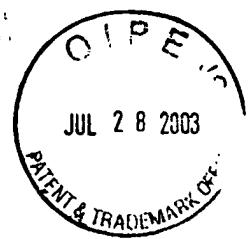
Conclusion

For the foregoing reasons, Applicant believes all the pending claims are in condition for allowance and should be passed to issue. If the Examiner feels that a telephone conference would in any way expedite the prosecution of the application, Applicant requests a call be made to the undersigned at 781-674-7852.

Respectfully submitted,


John M. Lucas
Reg. No. 43,373

**29 Hartwell Avenue
Lexington, MA 02421
(781) 674-7852 ph**



ISOLATION OF NUCLEIC ACID

RELATED APPLICATIONS

5 This application is a 371 of PCT/GB00/01684, filed 05/02/2000, which claims priority to patent applications UNITED KINGDOM 9910154.5, filed 04/30/1999, UNITED KINGDOM 9910157.8, filed 04/30/1999, UNITED KINGDOM 9910156.0, filed 04/30/1999, and UNITED KINGDOM 9910158.6, filed 04/30/1999, each incorporated herein in their entireties.

10

FIELD OF THE INVENTION

15 The present invention relates to a method for isolating RNA from a sample and to a kit for use in such a method.

Background to the InventionBACKGROUND OF THE INVENTION

20 The process by which RNA is separated from other molecules, in particular other cellular components such as proteins, carbohydrates, lipids and DNA is widely known and described in the literature. It is a key process in the understanding of gene function and structure and

25 drug development. Furthermore nucleic acid based diagnostic procedures for detecting RNA viruses such as HCV and HIV absolutely require the viral genomic RNA to be isolated in an intact and relatively pure form. In order to separate RNA in an intact and pure form

30 from other biological material it is necessary to rapidly inactivate all ribonucleases that are present and separate the RNA based on a biophysical property unique to the RNA. However, due to the similar physical characteristics of RNA and DNA, RNA preparations are

35 frequently if not always contaminated with DNA, leading

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to major difficulties in the analysis of results.

Currently mRNA is separated from genomic DNA by using a method based on oligo (dT) interacting with the 5 poly A tail (Aviv and Leder., Proc. Natl. Acad. Sci. USA. 69, 1408-1412 (1972). However, A-rich DNA sequences are co-purified with this method leading to mRNA contaminated with A-rich DNA. Alternatively, nucleases that are specific for either RNA or DNA can 10 be employed. Such highly purified enzymes are costly to use and frequently require removal before the nucleic acid can be used. For example RNase free, DNase must be removed by phenol extraction or heat inactivation otherwise it will destroy for example PCR primers or 15 other DNA based reagents in all post-nuclease reactions. Another method is to use a mixture of phenol, chloroform, isoamyl alcohol (50:49:1); whereby DNA preferentially partitions into the organic phase whilst RNA remains in the aqueous phase. This method at best still leaves a 20 significant amount of DNA contaminating the RNA and hence is of limited practical use. Another method is the TRI reagentTM-REAGENTTM (Molecular Research Centre, Inc) that allows the simultaneous separation of DNA, RNA and 25 proteins. However, it requires careful separation of different phases from each other and subsequent centrifugation steps whilst not assuring complete separation of DNA from the RNA due to the difficulty of pipetting small volumes of liquid without cross-contamination. Another method is anion exchange 30 chromatography which can separate RNA from DNA but the expense and difficulty of setting up the column as well as the restriction to purifying only small nucleic acids precludes its use from the majority of laboratories. Numerous methods to purify RNA are

described in general texts (Jones et al., (1994) in RNA Isolation and Analysis. Bios. Oxford., Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH.).

5 ~~Summary of the Invention~~ SUMMARY OF THE INVENTION

The present invention provides a preparative method for isolating RNA comprising an oligo- or polynucleotide from a sample, which method comprises:

- (a) treating the sample with a reactant capable of 10 covalently modifying the 2'-OH position of the ribose rings of the RNA under conditions so that a proportion of the 2'-OH positions of the ribose rings bear a substituent; and
- (b) preparing isolated RNA therefrom by separating 15 material containing the substituent from the sample on the basis of a property of the substituent.

The RNA may be mRNA, tRNA, rRNA, viral RNA, viroid RNA, synthetic RNA such as chemically synthesised or *in* 20 *vitro* transcribed forms, or any other form of RNA, such as hnRNA. The RNA may be a mixture of different types of RNA and may be in single- or double-stranded form, linear or circular and contain internal regions of secondary and tertiary structure such as is commonly found in 25 tRNA. According to the present invention an oligonucleotide generally has a sequence of up to about 80 bases and a polynucleotide generally has a sequence length of more than about 80, preferably more than about 100 bases. A preferred length for a 30 polynucleotide is at least 1000 bases.

The mRNA may or may not have a cap and/or poly A tail. The RNA used in the present invention is preferably

naturally-occurring. A naturally-occurring RNA according to the present invention typically comprises a nucleotide sequence which is found in nature and which has a structural function or generally encodes a 5 polypeptide having biological activity, or such a nucleotide sequence which is modified, for example to alter in some way the biological activity of the polypeptide encoded thereby. Whilst the naturally- occurring RNA is preferably obtained by transcription 10 from a suitable RNA or DNA template, itself usually naturally-occurring, in some cases the naturally- occurring RNA can be obtained synthetically. RNA according to the present invention does not encompass simple homopolynucleotides (poly A, poly U, poly G and 15 poly C) which can be generated synthetically but are biologically non-functional.

The naturally occurring RNA can be derived from a biological material such as bacteria, viruses such as 20 those causing infection in humans, animals or plants, viroids, or cells such as fungal, animal and plant cells.

An important aspect of this invention is modification of mRNA, rRNA and viral RNA since they are of major 25 scientific and clinical interest and serve as a good example of the problems encountered when manipulating RNA. The invention further provides methods for obtaining intact full-length copies of mRNA, rRNA, viral RNA and other types of RNA isolated from 30 cellular sources or extracellular fluids that demonstrate increased stability in conditions that would otherwise destroy a major fraction of the unmodified RNA.

Measuring the percentage modification of RNA
35 Due to the polymeric nature of RNA, it is difficult

to measure its molecular weight above 100 nucleotides using mass spectrometry because a large amount of RNA degradation occurs during the analytical process.

However, RNA (tRNA) up to 142 nucleotides (Nordhoff et al., (1993) Nucleic Acids Res. 21:3347; Gruic-Sovulj et al., (1997) Nucleic Acids Res. 25:1859; Tolson and Nicholson (1998) Nucleic Acids Res. 26:446) and double stranded DNA up to 500 base-pairs (Bai et al. (1995) Rapid Comm. Mass Spectrom. 9:1172; Taranenko et al., (1998) Nucleic Acids Res. 26:2488; Ausdall and Marshall (1998) Anal. Biochem. 256:220) have been measured using MALDI mass spectrometry (for reviews see; Smith (1996) Nat. Biotech. 14:1084; Murray (1996) J. of Mass Spectrom. 31:1203. Phosphate (Schuette et al., (1995) J. Pharm. Biomed. Anal. 13:1195; Sinha et al., (1994) Nucleic Acids Res. 22:3119) and chemically modified oligonucleotides (Potier et al., (1994) Nucleic Acids Res. 22:3895) have also been measured using mass spectrometry.

Although there is a molecular weight limitation to a few hundreds of nucleotides when using mass spectrometry, it provides a simple, automated means to accurately determine the exact molecular weight and therefore the percentage modification of a polynucleotide.

Optimisation relies on a number of factors such as the type of mass spectrometry being carried out (electro-spray, MALDI-TOF etc), the method used to purify the modified RNA from the modification reaction, the size of the polynucleotide, the ionisation matrix used, the method used to remove cations from the RNA, positive or negative ion mode and the voltage strength used (Fenn et al., 1989) Science 246:64). Capillary high performance liquid chromatography can be used prior to mass spectrometry of RNA because desalting and other purification steps are not required prior

to ionisation (Taniguchi and Hayashi (1998) Nucleic Acids Res. 26:1481).

To measure the molecular weight and hence the percentage modification of polynucleotides consisting of thousands of nucleotides requires a different approach. In certain situations where it is preferable to measure the percentage modification of the polynucleotide using more precise means a degradative step may be employed followed by an analytical process. It is expected that degradation of the modified polynucleotide using chemical or enzymatic means will, depending on the method used leave the 2'-OH modification attached to the ribose sugar allowing the amount of modification to be established by mass spectrometry or high performance liquid chromatography (HPLC). HPLC and gas chromatography analysis of nucleotides has been described (Gehrke and Patel (1977) J. Chromat. 130:103; Iwase et al., (1975) J. Chromat. 106:213; Kemp et al., (1982) J. ~~chromat.~~ Chromat. 241:325).

In order to establish the percentage of nucleotides that are modified, degradation of the polynucleotide should follow the modification reaction. Methods have been described for enzymatic cleavage methods employing ribonucleases RNase T1, RNase A, RNase U2, RNase PhyM, RNase CL3, nuclease S7 and cusativin, chemical cleavage methods using sulfuric acid (Jones et al., (1994) RNA Isolation and Analysis, chapter 3, Bios Scientific Publishers, Oxford) and physical methods using post source decay (Hahner et al., (1997) Nucleic Acids Res. 25:1957; Taniguchi and Hayashi (1998) Nucleic Acids Res. 26:1481; Kirpekar et al., (2000) RNA 6:296).

It will be understood that the 2'-OH modification may inhibit degradation of the polynucleotide. However, by empirically determining the sensitivity of the modified RNA to a range of conditions it will be should be possible in most cases to select conditions that are suitable for chain cleavage. For example, it has been found that acetylated RNA is readily cleaved by nuclease Bal 31. Whilst alkali cleaves acetylated RNA it also results in acetyl cleavage so unless the amount of cleaved acetyl groups is measured by mass spectrometry, acetylated nucleotides will not be detected. For example, acid cleavage of the modified polynucleotide can be used for base sensitive modifications, whilst base cleavage can be used for acid sensitive modifications. It will also be understood that other degradation products such as dinucleotides, trinucleotides etc will also be suitable for measuring the percentage modification of the polynucleotide. Whether it is the nucleotide, dinucleotide or larger fragments that are being measured, in each case it is the ratio of the number of fragments bearing a modification compared with the number of fragments not bearing a modification that provides the percentage modification.

Other methods that are capable of measuring high molecular weight RNA such as analytical ultracentrifugation to find the sedimentation coefficients (Svedberg units) are imprecise, require large amounts of starting material and are dependent on the conformation of the RNA (for review, see Jones et al., (1994) RNA Isolation and Analysis, chapter 3, Bios Scientific Publishers, Oxford).

Despite these drawbacks, analytical ultracentrifugation using denaturing sucrose or isokinetic gradients may be useful to measure very large molecular weight changes in abundant RNA samples.

It is now much more common to measure the molecular weight of polynucleotides using electrophoretic separation in polyacrylamide or agarose gels.

10 Detailed descriptions of the preparation, use and handling of electrophoresis gels is described in several publications (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH; Jones (1995) Gel Electrophoresis: Nucleic Acids Essential

15 Techniques, Wiley). Denaturing gels are preferred to non-denaturing gels because they reduce conformational effects providing a means to measure the true molecular weight of the linear polynucleotide (Jones (1995) Gel Electrophoresis: Nucleic Acids Essential Techniques, page 47, Wiley). There are a variety of denaturants that can be used such as DMSO (50-90%), glyoxal (10-30%), formaldehyde (3% w/v), formamide (50-98%), heat (60-80°C), methyl mercuric hydroxide (3-5mM), sodium

25 iodoacetate (10mM), 2-pyrrolidone (5%) and urea (6-8mM). It is known that incomplete denaturation of the polynucleotide leads to anomalous migration so that more than one denaturing condition may be required such as 8M urea plus 5% pyrrolidone or 8M

30 urea run at 60°C (Rosenblum et al., (1997) Nucleic Acids. Res. 25:3925) Capillary electrophoresis provides an excellent means to carry out such molecular weight determinations and suitable methods have been described for RNA (Engel and Dieguez-Lucena (1993) Nucleic Acids Res. 21:759).

Comparative measurements of polynucleotide migration between different gels is difficult because the distance migrated is dependent on the buffer used, gel 5 concentration and temperature. Therefore it is preferred that comparisons be made with both molecular weight standards and sample polynucleotides in the same gel. It is also known that certain percentages of the sieving matrix such as polyacrylamide or agarose are optimal for 10 certain lengths of nucleic acids and above a certain percentage of acrylamide or length of polynucleotide (the exclusion limit) separation as a function of length does not occur. Therefore, measurements of the molecular weight should be made within such known limits (Sambrook 15 et al., (1989) Molecular Cloning: A Laboratory Manual, CSH; Jones (1995) Gel Electrophoresis: Nucleic Acids Essential Techniques, Wiley).

It has been found that using a 20 cm 6M urea 5% 20 polyacrylamide gel that a 250 nucleotide acetylated RNA runs approximately 20 mm apart from the non-modified form as a tight band. Therefore, measuring the amount of modification of smaller modifying groups than acetyl (42 daltons) should be feasible. The acetylated RNA also 25 runs at a position predicted to be 100% modified as compared with RNA size markers.

It is common practice to be able to separate in a denaturing sequencing gel DNA polynucleotides differing 30 by as little as 1 nucleotide in a total length of 500 nucleotides i.e. by 0.2% or less difference in length (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH.). It is therefore reasonable to expect that accurate measurements can be made for the molecular 35 weight of RNA polynucleotides in their modified and

unmodified forms when the modifying group is large, for example 28 daltons for formyl and 42 daltons for acetyl. Measuring the percentage modification with other modifying groups may also be possible providing that the 5 molecular mass increase as a result of the modification is sufficient. For example, halogenation of the 2'-OH group should be readily measured for chlorine (35.4 daltons) and bromine (79.9 daltons) substitution of the 2'-OH group.

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Calculating the percentage modification may be carried out by measuring the migration of known RNA size markers in a gel such as a 6M urea-5% acrylamide sequencing gel and plotting migration (mm) versus molecular weight 15 (daltons) to obtain a standard curve. Because the exact, molecular weights of all the markers are known, it is straight forward to graph the relationship between the mobility in the gel versus the known molecular weight of each marker. The percentage modification for an RNA of 20 known length with a known mass for the modifying group can then be readily calculated by comparison with this standard curve.

An alternative means to determine the percentage 25 modification is to use a radioactively labelled reactant such as ^{14}C or ^3H acetic anhydride to modify RNA and then determining the molar ratio of radioactive acetyl groups to nucleotides in a known amount of RNA sample. If the molar ratio is 1:1, then 100% of the 2'-OH groups are 30 modified. It will be understood that radioactive isotopes can be incorporated into a wide variety of reagents.

Regiospecificity of the reaction can be determined by subjecting an identical sequence of DNA (or preferably

single stranded DNA bearing uracil as a replacement to thymine), to identical reaction conditions as used for RNA. It is expected that the DNA is not substantially modified as measured by incorporation of radioactivity, 5 gel electrophoresis mobility, mass spectrometry, HPLC or any other analytical means used if the reaction is regiospecific for the 2'-OH group.

Modification at the 2'-OH position is preferably 10 substantially regiospecific. Thus, there is preferably substantially no modification of the bases, phosphodiester bonds and/or any other position within the RNA chain. In this way, the polynucleotide retains important properties of the RNA. For example, 15 advantageously, the polynucleotide is preferably modified so that a single strand of the polynucleotide is replicable by a nucleic acid polymerase to generate a second strand of polynucleotide complementary to the single strand.

20 The modification at the 2'-OH position may be such that the entire OH of the 2'C of the ribose ring is replaced by a reactant group R as in 2'-R or by OR having 2'-OR where the -O- group may or may not originate from the 2'-OH group. Accordingly, the substituent at the 2'-OH 25 position in this case is R or OR respectively.

Various reactants or reactant combinations may be used, optionally in the presence of a catalyst, to provide these substituents, as described in further detail in the 30 Examples below. Advantageously, the reactant comprises an acid anhydride, an acid halide, an acyl cyanide, a carboxylic acid or an N-acylimidazole, an alkoxyalkyl halide, an alkylthioalkyl halide, an alkoxyalkoxyalkyl

halide, a trialkylsilane halide or a trialkylsilane imidazole. Each of these reactants participates in a modification (e.g. acylation, modification with an ether-containing group or with a silane) reaction with the RNA.

- 5 Preferably, the reactant is attached to a solid phase such as with benzoyl chloride polymer bound beads (BCPB) so that the modification reaction at the 2'-OH group covalently links the RNA to the solid phase. Under these reaction conditions, the reaction medium may further
- 10 comprise an acylation catalyst. For example, where the reactant comprises an acid anhydride, this may be reacted with the RNA in the presence of a catalyst such as a fluoride ion or amino pyridine. As a further example, where the reactant comprises an acid chloride or N-
- 15 acylimidazole, the reactant may be reacted with the RNA in the presence of an amino pyridine. As a further example, where the reactant comprises a carboxylic acid, this may be reacted with the RNA in the presence of a dehydrating agent or a catalyst, such as an isocyanide
- 20 catalyst. A preferred aminopyridine catalyst is dimethyl aminopyridine (DMAP).

The organic solvent used in the reaction medium of the present invention may comprise an organic base and may

- 25 comprise an organic solvent in which is dissolved the organic base or, in another embodiment, may be the organic base itself. It is preferred that the reactant is soluble in the organic solvent. In a preferred embodiment the reaction medium further comprises water. In this way
- 30 RNA to be modified may be conveniently added to the organic solvent as an aqueous solution of RNA. Typical organic solvents include alkanes such as hexane and pentane, pyridine, acetonitrile, dimethylformamide, dichloromethane, acetone, diethyl ether, benzene,
- 35 chloroform, ethyl acetate, light petroleum,

tetrahydrofuran, carbon tetrachloride, dichloroethane, dioxane, carbon disulphide, nitromethane, dimethyl sulphoxide, hexamethylphosphoric triamide and toluene. Typical organic bases include pyridine, triethylamine, 5 trimethylamine, diisopropylethylamine, *N,N*-diethylaniline, *N,N*-dimethylaniline, 1,5-diazabicyclo (4,3,0) non-5-ene (DBN), 1,8-diazabicyclo (5,4,0) undec-7-ene (DBU) and *N*-methylmorpholine. Triethylamine ($\text{CH}_3\text{CH}_2)_3\text{N}$ is a stronger (non-aqueous base) amine base 10 than pyridine, aniline, diethylamine or trimethylamine but less so than pyrrolidone. It is one of the strongest amine bases. A preferred organic base which acts as a solvent is triethylamine (TEA). Where a catalyst is to be used, it is convenient for the catalyst to be soluble in 15 the organic solvent as well. The water and the organic solvent may form different phases in the reaction medium. For example, the water and the organic solvent may be immiscible with one another and form phases which will separate upon standing. Where there is 20 more than one phase, the RNA may be reacted with the reactant under conditions of phase transfer catalysis.

The amounts of water and organic solvent may be varied and will depend to some extent upon the particular 25 organic solvent/base/catalyst system to be used. Advantageously, the reaction medium comprises at least 50% organic solvent, preferably at least 80%, more preferably at least 90% and more preferably at least 95% v/v. Typically, the ratio of water:organic solvent is in 30 the range 1:50 to 1:10, preferably around 1:20.

It should be noted that if the percentage of 2'-OH groups modified is low, then the remaining sites in the chain bearing non-modified 2'-OH groups will be sensitive to

degradation. Degradation may be caused during the reaction if basic solvents such as triethylamine, ethyldiisopropylamine or pyridine are used. Low percentage modification may be caused for a variety of 5 reasons. Bulky modification reagents such as pentanoic anhydride react more slowly than smaller reactants such as propanoic anhydride, possibly because of steric hindrance between the RNA polynucleotide and the reactant. It would be expected that even larger 10 reactants such as octanoic anhydride or oleic anhydride or those reactants that react relatively slowly would also lead to partial 2'-OH modification. In some cases, partial 2'-OH modification is desired because the RNA retains many of its original biophysical properties. 15 Partial 2'-OH modification can be favoured over complete modification of the RNA by using large and bulky reactants, increasing the amount of RNA added, adding a competing reactive group, removing the catalyst, reducing the temperature and/or time of reaction or the 20 amount of reactant present.

When the reactant itself is immobilised such as with benzoyl chloride polymer bound (BCPB) beads, it is unlikely that all the 2'-OH groups of a RNA polynucleotide 25 will be modified because the RNA will be spatially constrained thereby restricting the other 2'-OH groups from contacting the other immobilised reactants. When partial 2'-OH modification is unavoidable or even desired, it is preferable to use reaction conditions that 30 do not lead to RNA cleavage at non-modified sites such as by using non-basic solvents. It has been found that RNA in an intact form can be immobilised on BCPB beads when non-basic solvents such as tetrahydrofuran or dimethyl sulphoxide are used. If further protection/modification

is desired, a second modifying reagent can be added during or subsequent to the first reaction thereby reacting and protecting the remaining 2'-OH groups. Alternatively, the secondary reactant could carry a label

5 such as a fluorescent group (for example isatoic anhydride) allowing a simple means to quantitate the amount of RNA bound to the bead by measuring the amount of label subsequently attached to the RNA-beads. An example of such a secondary modification is given in the

10 Examples.

In the absence of a catalyst, the reaction time is generally from 20 to 60 mins. In the presence of the catalyst, the reaction proceeds more quickly, the

15 reaction time generally being completed within about 20 seconds.

On a vol/vol basis it is found that the ratio of reactant to reaction medium (especially acetic anhydride

20 triethylamine/DMAP) is preferably in the range 1:200 to 1:10, more preferably around 1:20. Too little reactant gives a partial reaction and too much makes the reaction difficult to control.

25 In accordance with the method of the present invention, RNA is isolated from the sample by separating material which contains the substituent which has been used to modify the 2'-OH position of the ribose rings. Accordingly, a substituent needs to be selected so as to

30 confer upon the RNA a property which the unmodified RNA does not possess. In one aspect, the substituent comprises an affinant capable of being recognised or bound by a partner such as a partner immobilised to a solid phase, or a protein. The affinant may be a

hapten such as biotin or fluorescein which could be recognised and bound by an antibody or another protein such as streptavidin. Another useful affinant comprises a primary amino group capable of

5 chemically reacting with an immobilised partner such as succinimide.

In one arrangement, the substituent comprises a hydrophobic substituent so that the RNA may be modified to render

10 it more hydrophobic for the purpose of isolation. Both DNA and RNA are relatively hydrophilic molecules. It is quite difficult to separate RNA from DNA because their physical characteristics are similar. By increasing the hydrophobicity of RNA relative to DNA it is possible

15 to improve the separation of the two types of nucleic acid. This is useful for either removing contaminating RNA from a DNA sample or removing contaminating DNA from RNA. For example it is important to remove bacterial RNA from a plasmid preparation prior to restriction enzyme

20 analysis so that small DNA fragments are not obscured by the co-migrating RNA during agarose gel electrophoresis. Alternatively it is important to remove traces of DNA such as genomic DNA or viral DNA from cellular or viral RNA. Such DNA contamination often leads to false

25 positives following RT-PCR amplification.

The hydrophobic substituent typically comprises a substituent, OR, wherein R comprises C₁-C₃₆ alkyl; C₁-C₃₆ alkenyl; C₁-C₃₆ alkynyl; C₁-C₃₆ haloalkyl; C₁-C₃₆

30 aminoalkyl; C₁-C₃₆ alkoxyalkyl; C₁-C₃₆ alkylthioalkyl; C₁-C₃₆ alkoxyalkoxyalkyl; C₁-C₃₆ haloalkoxyalkyl; C₁-C₃₆ aminoalkoxyalkyl; C₆-C₃₆ aryl; C₆-C₃₆ alkylaryl; C₆-C₃₆ arylalkyl; C₆-C₃₆ arylalkenyl; C₁-C₃₆ alkanoyl; C₁-C₃₆

35 alkenoyl; C₁-C₃₆ haloalkenoyl; C₁-C₃₆ haloalkanoyl; C₂-C₃₆ haloformylalkanoyl; C₁-C₃₆ C₁-C₃₆ aminoalkanoyl; C₁-C₃₆

azidoalkanoyl; C₁-C₃₆ carboxyalkanoyl; C₁-C₃₆
carboxyalkenoyl; C₁-C₃₆ carboxyalkynoyl; C₁-C₃₆
alkylaminoarylalkanoyl; C₁-C₃₆ alkoxy carbonyl; C₁-C₃₆
alkenyl oxy carbonyl; C₁-C₃₆ alkylsulfonyl; C₆-C₃₆
5 arylalkanoyl; C₆-C₃₆ arylalkenoyl; C₆-C₃₆ aryloxyalkanoyl;
C₆-C₃₆ alkylarylalkanoyl; C₆-C₃₆ haloarylalkanoyl; C₆-C₃₆
aminoarylalkanoyl; C₁-C₃₆ alkylsilanyl; C₁-C₃₆
trialkylsilanyl or C₁₂-C₂₈ diarylphosphano; or a
substituent R', wherein R' comprises C₁-C₃₆ alkyl; C₁-C₃₆
10 alkenyl; C₁-C₃₆ alkynyl; C₁-C₃₆ haloalkyl; C₁-C₃₆
aminoalkyl; halo; amino; C₁-C₃₆ alkylamino; C₆-C₃₆ aryl;
C₁-C₃₆ alkylaryl or C₁-C₃₆ arylalkyl.

In one embodiment, the hydrophobic substituent comprises
15 a C₄-C₇ carbon chain or ring, preferably one which arises
where the reactant comprises butyric anhydride, pentanoic
anhydride, hexanoic anhydride or benzoic anhydride. In
this case, it is preferred that the proportion of 2'-OH
groups bearing the substituent is at least 10%.

20 Alternatively, the hydrophobic substituent may comprise a
C₈-C₁₂ carbon chain or ring in which case it is preferred
that the proportion of 2'-OH positions bearing the
substituent is in the range 1 to 10%. As a further
25 alternative, the hydrophobic substituent may comprise a
C₁₂-C₃₆ carbon chain or ring more preferably a C₁₂ to C₂₄
carbon chain or ring. In this case, it is preferred that
the proportion of 2'-OH positions bearing the substituent
is up to 1%. Thus, whilst modification of up to 1% may
30 provide a sufficiently hydrophobic modified RNA where a
long chain length of substituent is used, substituents of
lower carbon chain length require a higher percentage,
perhaps in the range up to 95%, to be effective.

35 A number of methods for isolating RNA from samples

treated in accordance with the present invention exist. For example, the step (b) of separating material containing the substituent from the sample may comprise contacting the treated sample from step (a) with a 5 hydrophobic solid phase so as to bind the material containing the hydrophobic substituent and optionally washing the material bound to the hydrophobic solid phase. The hydrophobic solid phase may comprise hydrophobic particles. This method may further comprise a 10 step of eluting the material bound to the hydrophobic solid phase by treating with a detergent, a chaotrope or a solvent, by lowering the salt concentration or by cleaving the substituent from the 2'-OH position of the ribose rings.

15 In a further embodiment, step (b) comprises treating the treated sample from step (a) with a lyotropic salt to aggregate the material containing the hydrophobic substituent as an RNA precipitate, and isolating the 20 precipitate. The lyotropic salt preferably comprises ammonium sulphate, an alkali metal chloride, magnesium chloride or calcium chloride.

25 In a further embodiment, step (b) may comprise treating the treated sample with a non-polar solvent to form the hydrophobic liquid phase which contains the material containing the hydrophobic substituent, and isolating the hydrophobic liquid phase. The non-polar solvent typically comprises pentane, cyclohexane, toluene, benzene, light 30 petroleum, xylene or hexane.

35 In a further embodiment, step (b) may comprise use of a membrane, such as a PVDF membrane, to separate the RNA. The addition of hydrocarbon chains to the 2'-OH position of RNA increases its hydrophobicity, that is its ability

to interact with a hydrophobic surface. The use of hydrophilic PVDF filters to purify RNA has already been described in U.S. Patent 5,958,677 (Sept. 99). However, this describes only the binding of RNA, which is

5 in its unaltered form is a weakly hydrophobic molecule. Modified RNA molecules of increasing hydrophobicity can be created, as discussed above, by adding acyl groups of increasing chain length. Hydrophobicity increases in the order: 2'-OH < formyl (-C(O)H) < acetyl (-C(O)CH₃) < propanoyl

10 (-C(O)CH₂CH₃) < butanoyl (-C(O)CH₂CH₂CH₃), i.e. according to the carbon chain length. Longer chain lengths than butanoyl were not tested but would be expected to follow the same trend. The increase in the strength of the hydrophobic interaction was tested by

15 binding RNA and modified RNA to a polyvinylidene fluoride (PVDF) membrane and subjecting the bound material to increasingly stringent wash conditions (see Examples).

20 The reagent modifying the RNA 2'-OH group may be either free in solution or attached to a solid phase. In a particularly preferred aspect of this invention, a solid phase is employed. In this embodiment, the solid phase is not the same as the hydrophobic solid phase

25 described above and is used as a reactant so that the solid phase itself is introduced as the substituent modifying the RNA (note the hydrophobic solid phase is employed only after the RNA is modified with a hydrophobic substituent). When using a solid phase, the

30 modification reaction will lead to the covalent attachment of the RNA to the solid phase. This provides a convenient means to immobilise RNA to a solid phase in order to purify or analyse it. For example, RNA will be preferentially bound to the solid

35 phase from a RNA, DNA and protein mixture such as a cell

lysate or serum.

Advantages of using RNA covalently bound to a solid phase include ease of manipulation, purification, automation

5 and compatibility with many important life science applications such as diagnostics and detecting gene expression. It is also a convenient means to limit the amount of modification occurring on the RNA polynucleotide because the reactive groups are spatially

10 restricted. RNA modified at only a few 2'-OH positions may retain important biological functions such as serving as a template for protein translation, hybridisation and serving as a template for a polymerase.

15 There are many potentially useful solid phase reactants (see review by Akelah and Sherrington (1981) *Chem. Rev.* 81:557) such as other carboxylic acid derivatives including acid halides and acid anhydrides. Benzoyl chloride polymer bound (BCPB) beads are commercially

20 available (Fluka, USA) with high densities of benzoyl chloride groups (approximately 2.1mmol/g of resin). RNA can be covalently attached to the benzoyl chloride solid support via an ester linkage between the BCPB bead and the 2'-OH group. Under appropriate reaction conditions,

25 the 2'-OH groups of the RNA will react with the benzoyl chloride and become covalently coupled to the solid phase. Due to the physical separation of the benzoyl chloride groups, it is highly unlikely that all (100%) of the 2'-OH groups of a polynucleotide will be modified,

30 rather only one or a few will be modified per polynucleotide. In order to immobilise RNA, it is sufficient that only one 2'-OH group reacts with the solid phase reagent.

Other solid phase reactive groups that may be suitable for immobilising RNA could include acid anhydrides. In the case of acid anhydrides it is important that the half 5 of the acid anhydride attached to the solid phase is the part that reacts with the 2'-OH group thereby immobilising it. Otherwise, the result will be modified RNA in solution. In order to overcome this potential problem, an asymmetric acid anhydride may be attached to 10 the solid phase so that the half of the acid anhydride attached to the solid phase reacts preferentially with the 2'-OH group resulting in immobilised RNA. Other examples include the use of cyclic anhydrides such as isatoic anhydride polymer bound (Cat 17344, Fluka, USA). 15 However, it is simpler to employ carboxylic acid derivatives such as the acid halides (e.g. benzoyl chloride polymer bound), acid cyanides (e.g. benzoyl cyanide polymer bound) or acid imidazoles (e.g. N-benzoyl imidazole polymer bound) because the outcome of the 20 reaction will be an ester linkage between the solid phase and the polynucleotide.

It will be understood that the solid phase reactant need not be limited to carboxylic acid derivatives, rather any 25 reactant that reacts with the 2'-OH group in a regiospecific manner is suitable. The amount of particles required to immobilise a given amount of RNA will depend on several factors such as the density of the reactive groups and the proportion of the reactive groups that 30 will react with the 2'-OH. Reactive groups that are buried, for example within the resin will be unable to react with a large molecule like RNA and they will therefore be effectively unreactive. The precise RNA binding capacity for a particular solid phase reactant

will require empirical tests. If steric hindrance occurs between the polynucleotide and the reactive group due to the proximity of the solid phase a linker or spacer may be used between the solid phase and the reactive group 5 such as $-(\text{CH}_2)_{12-16}$.

The solid phase may consist of an inorganic particle, a polymeric linear, globular or cross-linked molecule or resin. It may be made of a variety of materials or 10 material composites such as acrylamide, agarose, cellulose, polyamide, polycarbonate, polystyrene, latex, glass and silicon. It may also possess specific properties that aid in the manipulation of the particle such as paramagnetic or magnetic properties, a diameter 15 allowing retention by a filter, an increased density that enhances sedimentation or separation by centrifugation or incorporate a tag aiding identification, capture or quantification of the particle.

20 The tag may be coloured, fluorescent, an isotope, an epitope for an antibody or a ligand for a protein such as streptavidin, or a protein such as an enzyme or antibody or an oligonucleotide. One purpose of the tag is to aid identification of the particle when particles from 25 several sources are combined. For example, if mRNA from tissue 1 is immobilised on particle type A, mRNA from tissue 2 is immobilised on particle type B and particles A and B are then mixed and added to a hybridisation reaction including for example a radioactive probe.

30 Following hybridisation, particles of type A and B are separated by means of a property unique to the tag and the radioactivity associated with each particle analysed to provide a measure of the relative gene expression in tissues 1 and 2. Other uses of the tag could include the 35 automated quantification and validation of the number of

particles in a vessel such as a 96-well plate.

The 'particle' or macromolecular structure may be incorporated into many forms such as a strip, a fibre, a 5 matrix, a membrane, a filter, a column, a bead, a resin, a vessel wall, a pipette tip, a gel or a plate or an etched silicon device.

The reactant may be covalently attached to the solid 10 phase using for example divinyl benzene (DVB) or associated by another means such as ionic interaction or hydrogen bonding so that in any case the RNA is firmly held to the solid phase during washing and analysis. However covalent attachment is preferred because there 15 will be less tendency for the reactant to dissociate from the solid phase. The solid phase reactant may be incorporated into a microdevice or vessel such as those made from etched silicon and the solution containing the RNA passed over or through the region containing 20 the reactant so that RNA immobilisation occurs in a specific region of the solid phase. This process may be automated in order to detect for example RNA viruses such as HCV and HIV in blood or body fluids. The immobilised sample might then be used for any number 25 of down stream applications such as hybridisation, RT-PCR, TMA or NASBA.

Alternatively, the solid phase reactant may be incorporated into a blood collection device such that 30 the RNA components of the blood are immobilised during the blood collection process. The solid phase reactant may also be coated or attached to a glass slide in many small (preferably less than 1mm²) discrete regions. A single RNA sample from, for example a tissue source may 35 be added (in a suitable solvent and catalyst) to one of

the discrete regions thereby localising the RNA to a discrete position of the glass slide. The process may then be repeated with other RNA samples until tens, hundreds or thousands of RNA samples are attached to as 5 many discrete regions of the slide as necessary. The slide could then be washed and subjected to hybridisation conditions with a labelled probe representing the sequence of interest such that multiple hybridisation events could be detected and with suitable instrumentation, 10 quantitated on a single slide. Such microchip applications could have very broad applications such as diagnostic or gene expression analyses.

For certain applications it may be useful to restrict 15 the size of the RNA that is immobilised. By using a macroporous resin with the reactant located within the pores, a size cut-off is established whereby only smaller RNA polynucleotides will be capable of accessing and therefore reacting with the reactant 20 whilst longer RNA polynucleotides are lost. This could be useful for determining the size of the RNA polynucleotides being analysed. Alternatively it may provide a means to specifically remove short RNA polynucleotides allowing the separation of longer 25 complete RNA polynucleotides that do not react with the solid phase. Separation of RNA based on size is currently carried out using either gel electrophoresis or column chromatography and this invention provides a simpler means to remove short polynucleotides from 30 solution. Commercial sources of beads with defined pore sizes are available for oligonucleotide synthesis.

Following immobilisation to the particle, the RNA may be further modified at the remaining 2'-OH positions in 35 order to endow it with additional desired properties such

as hydrophobicity, fluorescence, radioactivity or nuclease protection. The RNA may also be used for a variety of quantitative assays such as hybridisation with labelled probes or serve as a template for RT-PCR.

5 In the latter case, it has been found that BCPB beads have the advantage of being compatible with both the components of the reverse transcription and PCR reactions. Other purification materials such as silica beads inhibit these reactions.

10

A marked advantage of using BCPB beads compared with silica beads to purify RNA is that there is no elution step required to separate the RNA from the solid phase. If silica beads are added to the

15 reverse transcription reaction they would bind both the template RNA, primers and a proportion of the enzyme leading to reduced or total inhibition of cDNA synthesis. It has been found that following RNA reaction, BCPB beads can be added directly into both
20 the reverse transcription and PCR reactions with no such inhibition. BCPB beads may also find utility for other types of reactions such as LCR and NASBA. Adding the beads to the reaction simplifies manipulation and ensures that all the captured RNA is transferred into the
25 reaction. The bead format could prove useful for automated systems in the diagnostic field. Thus BCPB beads are especially preferred in the present invention.

Solvents

30 When RNA is modified using acetic anhydride in a basic solvent such as triethylamine with DMAP, the modification of the 2'-OH groups proceeds so quickly that base catalysed cleavage of the RNA is insignificant. However, when the reactant is attached to a solid phase such as
35 the BCPB, it is unlikely that all the 2'-OH groups can be

modified due to the physical immobilisation of the reactant. Therefore, even after extensive incubation times, the majority of the 2'-OH groups will not be modified and are therefore susceptible to cleavage by the 5 basic solvent. It is very important that the solvent used does not lead to RNA polynucleotide cleavage. Preferred non-basic solvents include tetrahydrofuran and dimethyl sulphoxide. Neither of these solvents results in RNA cleavage yet both the RNA and catalysts such as DMAP 10 readily dissolve in it.

It was found that some solvents increased the amount of BCPB bead sedimentation under unit gravity thereby decreasing the contact between the bead and solvent 15 bearing the RNA. Both THF and DMF allowed the beads to be well dispersed in solution whilst water, DMSO, pyridine, triethylamine and ethyldiisopropylamine (EDPA) promoted sedimentation. This may be an effect of both density differences between the bead and solvent and 20 hydrophobic interactions between the beads themselves. Preferred beads increase the contact between reactant and the solvent containing the RNA.

Amount and size of beads

25 It was found that adding a large excess of beads reduced the amount of RNA bound to the BCPB. For 50ng of RNA in 1 μ l of water, added to 80 μ l of THF/DMAP (5mg/ml) adding more than 1.5 mg of BCPB reduced the percentage of RNA bound from 74% to 48% (22mg BCPB). It was found that 30 during post-reaction purification of the BCPB beads that they tended to clump and stick together in aqueous solution making washing the beads difficult. This problem

was overcome simply by stopping the reaction with 3 reaction volumes of 70% ethanol and briefly mixing before washing with aqueous solutions. Preferred bead sizes are those that provide a maximum surface area for reaction to 5 occur with the RNA. Buried reactive groups will not be accessible to the RNA, therefore beads and particles with a small diameter (large surface area to volume) are preferred. However, in order to collect the beads from the liquid phase it is necessary that they are not so 10 small that they cannot be easily pelleted by centrifugation, collected by filtration or selected by other means. Commercially available BCPB beads with a mesh size of 100-300 are readily pelleted using a centrifugal force of 1500g for 5sec.

15

Length of RNA suitable for RT-PCR

Due to the physical separation of the reactive groups on the solid phase, a single RNA template may be linked to the solid phase via only one or a few 2'-sites. If 20 the RNA template is relatively long and the sequence to be amplified relatively short, there will only be a small number of RNA templates that are attached within the sequence to be amplified. These templates will probably not be copied by the enzyme because the 2'- 25 modification may block the passage of the enzyme. The HCV RNA genome is approximately 10,000 nucleotides long, if the PCR primers span a region of 100 bp, only 1% of the polynucleotides will not amplify due to the 2'- modification. However, if the RNA template is shorter, 30 for example 1000 nucleotides and the PCR primers span 1000 bp, then none of the RNA will serve as a template for RT-PCR. It is therefore preferable to PCR amplify only short regions of the RNA. Furthermore, the reverse transcriptase primer should be as close to the 3' PCR 35 primer as possible, preferably being of identical

sequence. However, for RT-PCR analysis of mRNA where the cDNA synthesis has to be primed using an oligo (dT) primer, it is preferable to use PCR primers that amplify sequences close to the 3' end of the mRNA. Alternatively 5 the RNA may be released from the solid phase using chemical or enzymatic deprotection, for example by alkali or KCN cleavage of the ester linkage before RT-PCR.

10 DNA Binding

Under specific conditions, some DNA will bind non-specifically to the beads probably through hydrophobic interactions with the BCPB bead. Such interactions can be reduced by using DMF, pyridine, 15 ethyldiisopropylamine (EDPA), triethylamine or DMSO as the reaction solvent instead of THF. Non-specific DNA binding can be removed by one or more washes in 10% SDS. However, DNA may react with BCPB beads covalently via either the 3' or 5'-OH groups. Less than 3% of a 20 labelled DNA sample became covalently attached when DMSO was used as a solvent for the BCPB beads compared with 14% with THF as a solvent.

Protein binding and diagnostic systems

25 In a similar manner to DNA, some protein will bind to BCPB. It was found that adding detergents to a THF solvent containing ³⁵S-labelled cellular protein enhanced the amount of protein binding to the beads. This may be caused by the detergent unfolding the protein and 30 therefore exposing more hydrophobic residues to the BCPB. The greatest enhancement was seen with a final concentration of 0.025% Tween/NP-40 TWEEN/NP-40 detergents, THF and protein were mixed and incubated 3 min at 22°C with 3mg of BCPB. Protein binding could be reduced by 50%

by pre-reacting the protein with acetyl chloride. Protein binding to BCPB was not reduced if the reactivity of the benzoyl chloride group was neutralised with water prior to addition of protein. This suggests 5 that protein binding is occurring non-specifically probably by a hydrophobic interaction.

When RNA was purified from solutions containing high concentrations of protein, it was found that a 10 proportion of the RNA tended to bind to the beads whilst complexed with protein in a non-specific manner. Such RNA could be readily removed by several washes in 10% SDS.

15 It was found that highest RNA binding to BCPB beads took place when the protein and RNA were added separately to the THF solvent rather than prior to mixing with the solvent. The beads were then added last and the reaction mixed and incubated.

20

A practical solution to non-specific protein binding to the BCPB beads may be the use of a protein removal step before the addition of the BCPB beads to the sample. For example this may consist of a protein pre-binding step: a 25 serum sample containing the RNA analyte may be added to, or passed through a protein binding surface such as a hydrophobic bead, membrane, filter or other protein capture surface and then the solution containing the deproteinised RNA added to the reactant-solid phase such 30 as BCPB beads. Suitable membranes that are known to capture proteins but not nucleic acids include 0.45 μ m pore size Immobilon-PIMMOBILON-P (hydrophobic polyvinylidene fluoride, Millipore, US). Alternatively, proteins could be separated from an RNA virus by

filtration whereby the proteins but not the virus particles pass through an ultrafiltration membrane with a nominal molecular weight cut-off of 30 000 -100 000 daltons such as ~~Centricon-100, Centriplus-100~~ CENTRICON-100, CENTRIPLUS-100 (Amicon, US). Filters with pore sizes above 0.01 μ m such as ~~Isepore~~ ISOPORE 0.05 μ m polycarbonate membranes (Millipore, US) should generally capture most virus particles from blood serum whilst allowing most proteins to pass through. Such collected virus particles could then be added to the reactant-solid phase such as BCPB beads.

The volume of a body fluid such as serum may be reduced prior to addition of the reactant-solid phase using a ~~Centrifree~~ CENTRIFREE filtration device. The retained sample containing protein and viral RNA could then be added to a solvent containing the reactant-solid phase such as BCPB beads.

Blood contains approximately 70mg/ml of protein and a large component of this is albumin and immunoglobulins. Means to reduce serum proteins include the addition of immobilised *S. aureus* protein A or thiophilic resin (Sigma, US) which bind immunoglobulins, a major component of blood protein. Protein A linked to a solid phase such as a bead or thiophilic resin would provide a facile means to reduce blood protein concentration.

Enhancing the Reaction

The RNA binding reaction may be enhanced by reducing the volume of the reaction to a minimum required to keep the RNA soluble and in contact with the beads.

Approximately 10 μ l of solvent is required to immerse 1-3 mg of BCPB. It was found that adding plasmid DNA to a final concentration of 25ng per microlitre enhanced RNA binding, possibly by increasing the apparent

5 concentration of the RNA. Other volume excluding compounds such as polyethylene glycol may also enhance RNA binding.

Addition of detergents and chaotropes

10

In order to increase specific RNA binding whilst reducing protein binding, detergents were added to a reaction containing BCPB beads in different solvents. It was found that the addition of a final

15 concentration of 1% SDS in DMSO or THF increased the amount of RNA bound to BCPB by 33% and 42% respectively whilst 1% SDS in DMF or toluene markedly

reduced RNA binding. It was also found that except for toluene, a final concentration of 1% SDS increased the

20 amount of 35 S labelled cellular protein bound to BCPB in DMF, DMSO and THF by 6.8, 2.3 and 1.85 times respectively that of a parallel reaction containing no added SDS.

EDPA, pyridine or water led to low levels of RNA binding.

25

It was found that addition of 150mM sodium perchlorate (final concentration) to 40 μ l DMSO containing 3mg BCPB beads, 10 μ g BSA and 20ng of radiolabelled RNA increased the amount of RNA bound by 2.3 fold. Even lower sodium

30 perchlorate concentrations of 1.5mM and 15mM (final concentration) increased RNA binding by 1.11 and 1.33 fold respectively. This effect was not caused by the high salt concentration because a parallel reaction containing 125mM NaCl instead of sodium perchlorate bound

30% less RNA.

The addition of sodium perchlorate also increases the amount of protein bound to the beads. 40 μ l of DMSO

5 containing 3mg BCPB beads was mixed with 1 μ l of 35 S labelled cellular protein with either 1 μ l of water or with 1.5mM, 15mM or 150mM sodium perchlorate (final concentration), protein binding increased by 0, 1.38 and 1.5 times respectively with sodium perchlorate.

10

However, 600mM urea or 400mM guanidine thiocyanate in THF reduced BCPB reactivity by 39% and 73% respectively. The addition of chaotropes and/or detergents is important because if RNA is to be

15 isolated intact from cells, blood or other biological material, it is necessary to inhibit ribonuclease activity. One way to do this is to use a slight excess of chaotrope and/or detergent and then add this to the solid phase reactant in a suitable

20 solvent system thereby immobilising the RNA. The solid phase may then be washed to remove contaminating proteins and DNA. Suitable chaotropes may include guanidinium chloride, guanidinium thiocyanate, sodium iodide, sodium perchlorate and sodium trichloroacetate.

25

An aliquot of BCPB stored at 22°C was opened over the course of 3 weeks and exposed to the air approximately 30 times. No reduction in reactivity was noted. BCPB is thus sufficiently stable for the present applications.

30 Water in excess of 7.5% vol/vol in THF reduces the reactivity of BCPB. The preferred water content is 3 μ l or less in 40 μ l of THF with 1mg of BCPB.

To reiterate, in addition to the preferred BCPB beads, the solid phase could include a particle, a bead, a membrane, agel, a slide or cover slip, an etched silicon surface, a fibre, a filter, a capillary, a 5 tube, a vessel or a multi-vessel plate such as a 96 well plate. It will be apparent that the use of a solid phase improves handling throughput and accuracy when the RNA ~~immobilisation~~immobilization and analysis is automated. In this context, paramagnetic particles are favoured for 10 their handling properties. Other preferred solid phases are those which comprise OH groups, e.g. compounds of silicon and oxygen, such as silica particles or a glass. Nucleic acid purification using silica beads is well known and widely ~~practised~~practiced both in diagnostic 15 and life science research. Methods of purifying RNA have been described in US patent 5,234,809. Briefly the method involves binding the nucleic acid sample to silica beads in a chaotropic agent such as urea and then washing the beads in a high salt wash before it is eluted into 20 water. The modified RNA used in the present invention has been tested for its ability to bind and be released from silica beads using a silica bead purification kit (Qiagen, Germany) and found to be particularly suited to this type of separation method (see Examples).

25 As mentioned above, according to US Patent 5,234,809, the purification of nucleic acids from biological samples involves mixing silica particles with a chaotropic salt containing the sample. Under these conditions the nucleic 30 acid binds reversibly to the silica. During attempts to find new methods to separate modified RNA from the reaction components, it has been discovered that modified RNA will readily bind to silica beads, e.g. in the presence of 95% organic solvent. This unexpected result 35 suggests that silica particles are suitable to bind

modified RNA from a variety of organic solvents.

Agents may be employed to aid in binding the RNA to the solid phase. Bifunctional acid halides, e.g. acid chlorides, are commercially available that have effectively two reactive groups per molecule. Reagents such as sebacoyl chloride ($\text{CLOC}(\text{CH}_2)_8\text{COCl}$), adipoyl chloride ($\text{CLOC}(\text{CH}_2)_4\text{COCl}$) and glutaryl chloride ($\text{CLOC}(\text{CH}_2)_3\text{COCl}$) can be used to immobilise RNA by covalent modification of the 2'-OH group with consequent attachment to a solid phase. The bifunctional acid chloride can either be reacted with the RNA and then with a solid phase, or preferably with a solid phase and then the RNA sample. In both cases, the solid phase is conveniently BCPB beads, or one which has hydroxyl groups such as glass and other silicon dioxide compounds. It is known that boiling concentrated HCl exposes hydroxyl groups on the surface of glass. Such prepared glass can then be reacted with an excess of the bifunctional acid chloride, the unreacted acid chloride is removed by washing in solvent such as THF and then the RNA sample added and allowed to react with the immobilised acid chloride, thereby becoming immobilised via the acid chloride to the glass solid phase.

Bifunctional acid chlorides are preferred that have an extended hydrocarbon spacer between each end of the molecule such as with sebacoyl chloride (Cat. No. 84848 Fluka, USA) because these are likely to reduce steric hindrance between the RNA and the solid phase and therefore improve the reaction.

Modification of RNA whilst bound to silica beads

Nucleic acids become bound to the surface of silica beads, therefore tests were carried out to find out whether RNA can be modified whilst bound to the silica

beads. Although it may be expected that some of the 2'-OH groups are protected from modification due to steric hindrance between the silica bead and the RNA, in practice, this effect was found to be undetectable 5 leading to substantially modified RNA (see Examples). When modifying with acetic anhydride, for example, compared with an RNA modification reaction with no acetic anhydride added, the modified RNA was substantially intact and migrated identically to an 10 acetylated RNA modified by the standard procedure. Modification, such as acetylation, of RNA can therefore occur whilst the RNA is bound or attached to a solid support or surface. This potentially offers advantages when high throughput of samples is 15 required or when RNA is purified using silica beads for example for diagnostic purposes from blood. This is therefore a preferred aspect of the present invention.

In a further aspect, the present invention provides a kit 20 for isolating from a sample RNA comprising an oligo- or polynucleotide, which kit comprises:

(i) a reaction system for modifying the RNA to form a modified oligo- or poly-nucleotide in which a proportion of the 2'-OH positions of the ribose rings bear a 25 substituent; and
(ii) a separation system for separating material containing the substituent from the sample, so as to isolate the RNA.

30 Preferably, the reaction system comprises:
(a) an organic solvent; and
(b) a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the RNA in the presence of the organic solvent. The organic solvent and reactant 35 are discussed in further detail above.

The present invention will now be described in further detail, by way of example only, with reference to the following examples.

5

General Procedure

A complex mixture of DNA and RNA polynucleotides from a cellular source is treated with a lipophilic acid anhydride such as butyric anhydride ($C_8H_{14}O_3$), pentanoic anhydride ($C_{10}H_{18}O_3$), hexanoic anhydride ($C_{12}H_{22}O_3$), heptanoic anhydride ($C_{14}H_{26}O_3$) or benzoic anhydride ($C_{14}H_{10}O_3$) as described. Longer chain lengths than heptanoic anhydride are not preferred for complete (100%) RNA modification because incomplete modification of the RNA chain has been observed, possibly due to steric hindrance between the RNA chain and the long chain anhydrides. Such reagents will modify the single terminal 3'-OH group and possibly the single 5'-OH or phosphate group of the DNA or RNA chain but all or substantially all the 2'-OH groups of the RNA, thereby strongly altering the physical characteristics of the RNA towards lipophilicity. However the DNA is modified at only one (3'-OH) or a maximum of two (one 5'-OH/phosphate and one 3'-OH) position(s) and therefore remains overall hydrophilic. This difference in lipophilicity then serves as the basis for separation of the DNA from RNA.

The hydrophobic RNA is preferably manipulated with silanised plasticware and/or with detergents such as 0.01% Tween-20, 0.01% NP40 or 0.1 Triton X-100 so that sample loss is reduced.

The capture surface or material for the modified RNA could be encapsulated within a centrifuge device

such as a microcentrifuge spin-column or tube insert. Alternatively, it could be encapsulated in a pipette tip such as those commonly used to measure 1 μ l, 10 μ l, 200 μ l or 1 ml volumes. In either case, capture, 5 washing and elution of the modified RNA is improved because the time required to separate the modified RNA from the contaminants is reduced.

Multiple RNA samples could be purified in parallel by the 10 use of devices bearing multiple capture surfaces. An example would be a 96-well plate whereby each well is hydrophobic and suitable for capture, washing and elution of one modified RNA sample. A further example would be a vessel or chamber suitable for the modification reaction 15 and in addition the capture, washing and elution of the RNA sample. In this way, both the modification and purification is carried out in the same vessel or chamber improving sample throughput and productivity. The modification, capture, washing and elution of the RNA 20 sample could be automated and involve robots.

Methods of treatment of the RNA are described in further detail in UK patent applications, nos. +9910154.5 entitled POLYNUCLEOTIDES published 30 June 1999 s 25 GB9910154A, 9910157.8 entitled POLYNUCLEOTIDES published 30 June 1999 as GB9910157A and 9910156.0 also entitled POLYNUCLEOTIDES). published 30 June 1999 as GB9910156A. Each of these copending patent applications was filed ~~on~~ 30 the same day as the present application 30 April 1999 in 30 the name of the same applicant. For example, Example 6 of each of these copending patent applications sets out one preferred method of modifying RNA involving a DMAP-catalysed acetylation reaction. The methodology in that Example may be modified using instead of acetic

anhydride, anhydrides of longer chain length including butyric or pentanoic anhydrides as set out in Example 54 of each of the copending applications. In addition, Example 1 of each of these copending patent applications 5 sets out a method of modifying a total cellular RNA population and selection of the mRNA fraction.

It will also be apparent to those skilled in the art that a crude cellular or tissue lysate consisting of RNA, DNA, 10 protein and lipids etc. may serve as the sample for the modification reaction. In this case, the reactant may modify not only the 2'-OH group of the RNA chain but also the hydroxyl bearing side chains of the amino acids tyrosine, serine or threonine of proteins. It will be 15 apparent that such a reaction is beneficial because it will lead to the deactivation of cellular nucleases and therefore allow the selection of RNA in a more intact form. Furthermore, cells could be disrupted in the presence of the reactant so that nucleases are immediately 20 inactivated on release from the cell and the RNA is immediately modified and therefore protected from any remaining nucleases. In this latter case, increased concentrations of the reactant in the reaction may be required in order to inactivate the nucleases fully 25 as well as to modify fully the 2'-OH groups of the RNA sample.

The treated DNA and RNA sample in a high salt buffer (e.g. 1-5 M ammonium sulphate, preferably 10 mM 30 phosphate, pH 7.0, 1.5 M ammonium sulphate) is passed over a hydrophobic column as is commonly used for reverse phased liquid chromatography. For example reversed phase packings based on silica may have bonded hydrocarbon chains of C4, C8 or C18 or others may be

based on polystyrene (e.g. POROS® and OligoOLIGO R3, PerSeptive Biosystems, USA) may be used under appropriate conditions. Further alternatives could include plastics such as polypropylene, polycarbonate, 5 polyvinylidene fluoride (manufactured under the tradename HYDROPHOBIC Hydrophobic Durapore, DURAPORE, (Millipore, USA) and PTFE. With all plastics those with a large surface area to volume are preferred in order to increase the effective hydrophobic binding surface. These 10 types of surfaces exist as beads of cellulose covered with butyl, octyl or phenyl groups (Cellufine Butyl, Cellufine Phenyl and Cellufine Octyl, (CELLUFINE BUTYL, CELLUFINE PHENYL and CELLUFINE OCTYL, Amicon, Mass, USA) and are well known in the art. Other bead compositions 15 could include silica matrix modified with polyethylene-imine (Si(SI PAE, Amicon, Mass, USA). After washing extensively with water to remove all traces of DNA, the RNA can be released by several methods. Firstly and preferably, the RNA can be deprotected by cleavage of 20 the modifying group with 50% ammonia treatment, 10-40mM KCN (final concentration) in 95% EtOH, K₂CO₃ in aqueous methanol or other conditions which are known to lead to the cleavage of the ester linkage (see Protective Groups In Organic Chemistry, 2nd edition, Ed. 25 T.W. Greene, Wiley-Interscience). The RNA can then be collected and purified further if required by, for example oligo (dT) selection of the mRNA.

Alternatively the RNA can be removed from the solid 30 support in its modified form using detergents such as 0.1% SDS, 0.002M Triton X-100™ TRITON X-100™ (Union Carbide Chemical and Plastics, Inc.), Nonidet P-40™ NONIDET P-40™ (Shell Oil Co.) or 1% Tween™ TWEEN™ (ICI Americas, Inc.) in aqueous solution. Or

alternatively by lowering the salt concentration (preferably 10 mM phosphate, pH 7.0) in the binding buffer to a point where elution is brought about.

Another approach is the addition of a chaotropic agent 5 such as ethylene glycol, urea, guanidine-HCl or thiocyanate salts. Solvents such as isopropanol, methanol, ethanol, DMSO or acetronitrile may also be used to elute the RNA up to a final concentration of 40% (v/v) in water.

10

The invention will now be described in further detail, by way of example only, with reference to the following Examples and the accompanying drawings, in which:

15

BRIEF DESCRIPTION OF DRAWINGS

FIGURE 1 shows RNA attachment strength on a PVDF membrane, plotting the % of remaining RNA on filter 20 against wash conditions; and

FIGURE 2 shows data relating to the release of RNA and acylated RNA from silica beads, plotting the % RNA released from silica beads against RNA type.

25

DETAILED DESCRIPTION OF THE INVENTION

Example 1

Use of lyotropic salts

30 Separation can be brought about using such commonly used salts as ammonium sulphate or sodium chloride (known as « lyotropic salts ») which are routinely used for separating proteins with varying hydrophobic

properties from solution by a process known as 'salting out'. Proteins with the greatest hydrophobic tendencies aggregate and precipitate out of solution at the lowest lyotropic salt concentration. By comparison with this

5 system it will be apparent that a specific salt concentration can be selected that will cause modified RNA to aggregate whilst the hydrophilic DNA will remain in solution thereby providing the basis for separation. The specific salt

10 concentration will depend on the particular modifying reagent used. For example, RNA modified with longer carbon chains which are therefore more hydrophobic such as heptanoic anhydride will aggregate at a lower salt concentration than those modified by shorter

15 chain lengths such as acetic or propionic anhydride. Other salts that may be used (in order of decreasing ability to cause RNA to aggregate) include RbCl, KCl, NaCl, CsCl, LiCl, MgCl₂ and CaCl₂.

20 Aggregates can be collected either by allowing precipitation to occur at unit gravity or by centrifugation at 1-5000 g in a bench top centrifuge. The modified RNA can then be collected and salts removed by washing with several washes of 70% ethanol.

25 Alternatively, salts can be removed by using a ~~Centricon~~ 50CENTRICON-50 column (Amicon, MA, USA) and washing with either water or 0.1% SDS. Following this purification procedure and, alternatively following the removal of the hydrophobic group, the

30 RNA may be used as a template for RT-PCR or in northern blotting.

Example 2

Partition Between Two Solvents

As another alternative separation procedure, modified RNA
5 with increased hydrophobic properties could be
simply purified from a complex mixture of proteins, DNA
and other cellular components by mixing and agitating
by use of a vortex etc. the mixture with appropriate
solvents such as (in order of decreasing polar property)
10 pentane, toluene, chloroform, THF, DMSO or methanol.
Under ideal conditions it would be expected that all
the modified RNA would partition into the
hydrophobic phase whilst non-RNA contaminants would
remain in the aqueous phase. Simple separation of the
15 hydrophobic phase by pipetting followed by ethanol
precipitation or evaporation of the solvent would provide
a highly purified source of modified RNA.

Example 3

20 Differential Interaction with Immobilised hydrocarbon chains

Direct interaction between RNA molecules bearing
hydrophobic groups and hydrophobic groups attached to
a solid support would provide an efficient means to
25 separate RNA from contaminants. Hydrophobic solid
supports include ethyl-, propyl-, butyl-, pentyl-, hexyl-
, octyl-, decyl and dodecyl-agarose affinity
chromatography media (Catalogue ref. AAF-8, Sigma-
Aldrich Chemicals). Interaction between the modified
30 RNA and the media should be stronger with longer chain
lengths attached to either the RNA or immobilised
support. By careful choice of the binding solution it is

possible to selectively bind RNA to the beads whilst the contaminants such as DNA are retained in the binding solution. The polarity of the solvent used for binding and washing, the type and concentration of 5 detergent, temperature of interaction and carbon chain length used will all influence the effectiveness of the purification.

60 μ l of ethyl-agarose or dodecyl-agarose beads (Sigma, USA) 10 were washed twice in 200 μ l of 1.7M ammonium sulphate and collected by centrifugation at 3000g for 5 seconds between washes. 40 μ l (20%) of the beads in of 1.7M ammonium sulphate were added to 300ng of isatoic anhydride labelled RNA. It was found that the fluorescent 15 isatoic anhydride modification could be used to monitor binding to the hydrophobic beads under ultra-violet light. On addition to the beads, fluorescent RNA moved from the solvent to the beads within a few seconds demonstrating interaction between the modified RNA and 20 the hydrophobic surface. It was also found that the interaction between the modified RNA and dodecyl-agarose was very strong: 1% ~~Tween/1% Triton~~ TWEEN/1% TRITON X-100, 6M urea, 100% ethanol or loading the sample in a well of an agarose electrophoresis gel and subjecting the bead- 25 RNA complex to 100V for 15min. failed to displace the labelled RNA from the dodecyl-agarose bead whilst 50mM sodium phosphate buffer removed most of the modified RNA from ethyl-agarose beads. An intermediate binding affinity between ethyl and dodecyl was found in the ease 30 of releasing modified RNA from propyl, pentyl and octyl- agarose. Overall, the strength of the hydrophobic interaction is proportional to carbon chain length increasing from ethyl, propyl, pentyl, octyl to dodecyl- agarose.

This demonstrates that as expected, the strength of the hydrophobic interaction between modified RNA and the hydrophobic bead is dependent on the chain length and

5 therefore hydrophobicity of the bead. The ease with which modified RNA may be removed from the hydrophobic surface is therefore a function of the hydrophobicity of both the modification at the 2' position of the RNA and the nature of the hydrophobic surface. Preferred chain lengths are

10 octyl and longer. Binding was found to take approximately 2 minutes and took place even in low salt binding buffers such as 50mM sodium phosphate buffer, pH 7.2 or water, although with water the amount of binding is reduced compared with 1.7M ammonium sulphate. It will be apparent

15 that RNA can be separated from other cellular components based on an initial modification reaction of the 2'-OH groups followed by selective hydrophobic binding to a hydrophobic surface, and where necessary subsequent release before use for hybridisation etc. This approach

20 could be useful for purifying RNA from biological samples such as cells for gene expression studies or viral RNA from biological material such as body fluids such as serum and cerebral spinal fluid.

25 Release of the modified RNA from the solid support is effectuated by the addition of;

- 1) a deprotecting agent such as alkali, ammonia or KCN that leads to acyl cleavage and therefore separation of the RNA from the hydrophobic groups,
- 30 2) use of a solvent with a low polarity such as pentane or toluene that will preferentially bind to the hydrocarbon side chains of the RNA and thereby disrupt the hydrophobic interaction with the solid support, or
- 35 3) a detergent such as SDS or TWEEN that is capable of disrupting the hydrophobic interaction with the solid

support.

4) addition of a Lewis acid such as MnCl₂ to cleave methoxyethoxymethyl ethers at the 2'-OH position, thus releasing the RNA from the hydrophobic surface.

5

Example 4

4

Immobilisation on BCPB beads with subsequent secondary modification reaction

10

2mg of BCPB beads were added to 40μl of THF and then 512ng of BMV RNA (Promega, US) in 2μl of water was added and briefly vortexed then incubated for 10min at 22°C.

15 1μl (10μmol) of acetic anhydride was then added in 20μl of THF containing 180μg of DMAP. The reaction was allowed to proceed for a further 5 min at 22°C and then stopped with 200μl of 70% ethanol and the beads collected by centrifugation at 3000rpm for 5sec and then washed a second time in 70% ethanol and twice in 200μl of

20 water before being resuspended in 20μl of water. The secondary reactant such as acetic anhydride or acetic-formic anhydride could also carry various labels such as 14C or 3H allowing the amount of RNA to be determined using a scintillation measurement of the

25 washed RNA-bead complex. Alternatively, the secondary reactant could be fluorescent such as isatoic or N-methylisatoic anhydrides or carry a label such as biotin permitting quantification of the amount of RNA bound to the bead. The secondary reactant would be expected

30 to modify at least 75% or more of the 2'-OH groups so that the RNA is protected from degradation from for example ribonucleases. However, the secondary modification could also provide a means for a second purification

step. For example the secondary reactant could contain a hydrophobic group or a ligand such as biotin for binding to a streptavidin bead. On release from the first solid phase such as BCPB beads the RNA would be specifically

5 bound to a hydrophobic surface such as dodecyl-agarose beads.

Example 5

Probe hybridisation to RNA immobilised on BCPB beads

10 100ng of BMV RNA (Promega, USA) in 1 μ l of water was added to 40 μ l of either THF or DMSO and then 3mg of BCPB beads added, mixed and incubated at 22°C for 15min, the beads were then washed once in 100 μ l of 70% ethanol, once in 100 μ l of 10% SDS and three times 100 μ l of water. The

15 beads were finally resuspended in 50 μ l of Church hybridisation buffer (0.5M NaPi pH7.2, 7% SDS and 1mM EDTA) containing a 32P dCTP radiolabelled probe complementary to the BMV RNA sequence (RNA 2, nucleotides 1-321). Following incubation for 2 hrs at 55°C, the beads

20 were washed twice in 500 μ l of 2 X SSC/0.1% SDS to remove non-hybridised probe and then the amount of radioactivity remaining on the beads was determined using a scintillation counter.

25 The probe can be branched or linear and labelled with a radioactive, fluorescent, coloured, affinity or protein label in a manner identical to standard hybridisation procedures. In this way, the amount of RNA complementary to the probe can be quantitatively determined. This could

30 be useful for example when determining the abundance of transcripts representing specific genes in different tissues. In this case, purified RNA from the tissue of interest could be immobilised onto the BCBP beads and complementary labelled probes hybridised. Unlike a

traditional dot blot (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH) the RNA is attached to beads and not a membrane, therefore the kinetics of hybridisation are improved (see Wilkins Stevens et al. 5 (1999) Nucleic Acids Res. 27:1719). Automation of bead handling could be enhanced by the use of paramagnetic particles. Other uses could include diagnostics where a RNA virus from, for example a body fluid such as serum, is immobilised on the BCPB bead or other solid phase 10 reactant and a probe representing a viral sequence hybridised to the RNA-bead complex. The amount of probe hybridised to the bead would indicate the amount of viral RNA in the starting material.

15 Example 6

Use of bifunctional acid chlorides to immobilise RNA
To 5 μ l of Qiaex QIAEX silica particles (Qiagen, Germany) in water was added 100 μ l of tetrahydrofuran (THF) and the particles washed once by centrifugation and then 20 resuspended in 40 μ l of THF containing 15 μ l (65 μ mol) of sebacoyl chloride and the reaction incubated at 22°C for 3hrs. The particles were then washed twice in 100 μ l of THF in order to remove unreacted sebacoyl chloride and then resuspended in 20 μ l of THF. The sebacoyl chloride at 25 this stage is covalently attached to the silica particles in such a way that there is one unreacted acid chloride group free to react with the RNA which is added to 20 μ l of the particles in 1 μ l of water. The reaction is allowed to proceed at 22°C for 30 minutes and then the particles 30 washed with 200 μ l of 70% ethanol and twice in 200 μ l of water. Non-basic solvents are preferred so that the RNA is not degraded during the course of the reaction.
Suitable solvents include THF, dimethyl sulphoxide and

dimethyl formamide. Catalysts such as DMAP, 4-pyrrolidinopyridine, other aminopyridine catalysts, tetrabutylammonium fluoride or tributylphosphine may be used in catalytic quantities during the reaction. The 5 covalently immobilised RNA can then be used for a variety of downstream applications such as RT-PCR amplification, hybridisation and NASBA,

Example 7

10 Purification of Acylated RNA using a PVDF Membrane

The following method was used. A radioactively labelled RNA transcript was produced using a Riboprobe RIBOPROBE kit (Promega, USA), T3 RNA polymerase, ³²P UTP and a 15 plasmid DNA template (pGEMEX-1 linearised with Hind III). Approximately 10,000 cpm of radiolabelled RNA (10 ng) was modified in separate reactions with acetic formic anhydride, acetic anhydride, propionic anhydride or butyric anhydride as follows.

20

Formylation: 5 µl of acetic formic anhydride was added to a 100 µl reaction containing 95 µl THF, 16 mg (195 µmol) 1-methylimidazole, and 10 ng radiolabelled RNA and the reaction incubated at 22°C for 10 min before purification 25 by ethanol precipitation.

Acetylation, propanoylation, butanoylation: A 20 µl reaction containing 19 µl triethylamine, 100 µg DMAP, 2 µl of anhydride and 10 ng of radiolabelled RNA was mixed 30 and incubated for 1 hr at 22°C. All modified RNA samples were purified by ethanol precipitation. Approximately equal amounts of RNA or modified RNA (5,000 cpm) was spotted onto a hydrophobic PVDF membrane (Amersham Pharmacia, UK) that had been pre-wet with methanol.

After briefly air drying the membrane, the radioactivity in each spot was measured before and after the following wash treatments, water 5 min at 22°C, water 5 min at 45°C, water 5 min at 55°C, 0.1% SDS 5 min 5 at 100°C, 0.1% SDS 20 min at 100°C. The amount of radioactivity remaining in each spot was quantitated using an Instant Imager (Hewlett Packard). The results are presented graphically in Figure 1. It is apparent that the butanoylated RNA had the greatest capacity to be 10 retained on the membrane as the following percentage of sample remaining after 20 min of boiling 0.1% SDS show. RNA 6.5%, formyl RNA 13%, acetyl RNA 21%, propanoyl 29% and butanoyl 89%. Although there is a clear linear increase in the percentage of sample retained as the 15 modification at the 2'-OH group increases in size from formyl to propanoyl, there is a much larger retention of sample with butanoylated RNA. In summary, over 93% of the RNA sample is lost whilst only 11% of the butanoylated RNA sample is lost after 20 min of boiling. Binding of 20 modified RNA to hydrophobic surfaces such as PVDF membranes or polystyrene beads is therefore a simple and realistic means to purify it from less hydrophobic contaminants such as DNA or carbohydrates. This may prove useful for purifying RNA from biological samples 25 such as blood or tissue samples for use in a diagnostic test.

Example 8

Binding of RNA to Silica Particles

30 Approximately 5,000 cpm of a 75 nucleotide RiboprobeRIBOPROBE radiolabelled (Promega, USA) RNA, formyl RNA (fRNA), acetyl RNA (aRNA), propanoyl and butanoyl RNA (pRNA and bRNA) was mixed with 10 µl of silica (Qiaex™)(QIAEX™) particles as described (Qiagen,

Germany) and then washed two times in 0.5 ml of buffer PE. The pellet was allowed to briefly air dry before adding 20 μ l of water and incubating for 5 min at 22°C. After the water was removed, the amount of cpm remaining 5 on the beads was measured and the results are shown graphically in Figure 2.

There was a proportional relationship between the length of the modifying group on the 2'-OH group and the 10 retention on the bead. Although 75% of the RNA was released from the beads, less than half the amount of the butanoylated RNA was released suggesting it is possible to purify modified RNA on the basis of the size of the modification. It was found that with longer incubation 15 periods at 50°C in 10 mM Tris-HCl (pH 7) it was possible to release over half (54%) of the butanoylated RNA from the silica beads.

Example 9

20 Modification of RNA whilst still bound to silica beads
The modification was carried out as follows. 1 μ g of 0.24 - 9.4 kb RNA ladder (Life Technologies) was mixed with 4 μ l of silica beads (QiaexQIAEX, Qiagen, Germany), mixed with 12 μ l of saturated sodium iodide and incubated 25 for 5 min at 22°C. The sodium iodide was removed and the washed beads added to 19 μ l of triethylamine, 100 μ g of DMAP and 2 μ l of acetic anhydride and the reaction incubated for 5 min at 22°C. The beads were then washed two times in 500 μ l PE buffer A and centrifuged 30 for 5 min at 13,000 rpm. The RNA was eluted in 5 μ l of water and analysed by agarose gel electrophoresis. Compared with an identical RNA modification reaction with no acetic anhydride added, the modified RNA was substantially intact and migrated

identically to a acetylated RNA modified by the standard procedure. Acetylation of RNA can therefore occur whilst the RNA is bound or attached to a solid support or surface. This potentially offers advantages when high 5 throughput of samples is required or when RNA is purified using silica beads for example for diagnostic purposes from blood.

Example 10

10 Purification of modified RNA from the reaction components using silica beads.

In order to purify the modified RNA from the reaction components the following procedure was used. A standard 20 μ l modification reaction containing 19 μ l 15 triethylamine, 100 μ g of DMAP, 2 μ l of acetic anhydride and 1 μ g of 0.24 - 9.4 kb RNA ladder (Life Technologies) was incubated for 2 min at 22°C and quenched by the addition of 20 μ l of ethanol. 4 μ l of silica particles (Qiaex, QIAEX, Qiagen, Germany) were added 20 and mixed for 5 min at 22°C. The particles were pelleted at 13,000 rpm for 5 min and washed once in 50 μ l PE and eluted in 10 μ l of 10 mM Tris-HCl (pH 7.5) at 22°C. Recovery of the acetylated RNA was estimated to be over 75%. The novel finding that nucleic acids can bind to 25 silica beads in 95% organic solvent suggests a general means to purify nucleic acids or products thereof from organic reactions or biological samples.

Example 11

30 BCPB beads RNA immobilisation reaction

2mg of BCPB beads were added to 40 μ l of THF and then 512ng of BMV RNA (Promega, US) in 1 μ l of water was added and briefly vortexed then incubated for 10min at 22°C.

The reaction was stopped with 200 μ l of 70% ethanol and the beads collected by centrifugation at 3000rpm for 5sec and then washed a second time in 70% ethanol and twice in 200 μ l of water before being resuspended in 20 μ l of water.

5

RT-PCR

One half (10 μ l) of the RNA-BCPB beads were added to a 20 μ l reverse transcription reaction containing the following final component concentrations: 200 mM Tris-HCl (pH 8.4 at 24°C), 75 mM KCl, 2.5 mM MgCl₂, 10 mM DTT, 1 mM dNTP's, 110 ng of BMV-R oligonucleotide primer (GAGCCCCAGCGCACTCGGTC) (SEQ ID NO: 1) and 100 units of the reverse transcriptase MULV Point Mutant (Promega, USA). The reaction was allowed to proceed for 20 min at 42°C and 10 min at 37°C. The cDNA/RNA BCPB bead complex was then washed four times in 200 μ l 100mM NaCl/ 10mM Tris HCl pH7.5, once in 200 μ l of water before resuspension in 10 μ l of water. The cDNA can then be used directly in a PCR reaction as follows.

15

The PCR was carried out in a final volume of 25 μ l with final concentrations of 15mM Tris-HCl pH 8.8, 60mM KCl, 2.5mM MgCl₂, 400 μ M each dNTP, 10 pmol of each primer BMV F (CTATCACCAAGATGTCTTCG) (SEQ ID NO: 2) and BMV R (GAGCCCCAGCGCACTCGGTC) (SEQ ID NO: 1) and 1 unit Taq DNA polymerase (Amersham Pharmacia Biotech, UK). 1 μ l of the bead complex was added per reaction. Cycle parameters were 94°C x 20 sec, 55°C x 20 sec and 72°C x 30 sec for 31 cycles. PCR products were visualised following agarose 20 gel electrophoresis and staining with EtBr. Excellent amplification of a 150bp BMV sequence was obtained using this method with product yields similar to the same amount of RNA free in solution.

Example 12

RNase treatment of BCPB-RNA

A large part of the RNA bound to BCPB can be cleaved
5 using ribonucleases such as ribonuclease ONE™ (Promega,
US) and RNases A. A BCPB-radioactive RNA
(Riboprobe, RIBOPROBE, Promega, USA) complex was mixed in
a 20µl reaction containing 1X RNase ONE buffer
(Promega, USA) and 1ng of enzyme for 30min at 37°C and
10 the amount of radioactivity released was counted using
a scintillation counter. Using either RNase A or
RNase ONE, 64-68% of the RNA was cleaved demonstrating that
the RNA is attached to the BCPB bead in a
conformation suitable for enzyme recognition. An
15 identical reaction with DNase I resulted in less than 5%
RNA release.

Example 13

Purification of RNA from a solution containing BSA

20 Serum albumin is a major component of blood. In order
to test the effect of serum albumin (BSA) on RNA
binding to BCPB beads, the following experiment was
carried out. To 20ng (1µl) of ³²P UTP radiolabelled
25 Riboprobe-RIBOPROBE RNA (Promega, USA) containing two
transcripts of 250 and 1500 nucleotides, was added
either 1µl of water, or 100ng, 1µg or 10µg of
acetylated BSA in 1µl of water and then mixed with
40µl of THF containing 1mg of BCPB beads. The four
30 reactions were mixed and incubated at 22°C for 3min, the
beads washed with 100µl of 70% ethanol and then with 50µl
of 20% SDS. The overall percentage of captured RNA was
similar for all three of the reactions containing BSA
(22-24%) compared with 46% for the reaction containing no

protein. This demonstrates that although the amount of RNA captured is reduced by half in the presence of protein, high protein concentrations (5 μ g/ μ l) are compatible with BCPB bead purification.

5

Example 14

Pre-reaction of the protein component with acid chloride
In order to reduce non-specific binding of protein to
the BCPB beads, the protein/RNA mixture was first
10 reacted with acetyl chloride. To 40 μ l of DMSO
containing 1 μ l of 6M sodium perchlorate, 1 μ l of
water, 1 μ l of serum and 1 μ l (20ng) of radiolabelled RNA
was added 1 μ l of acetyl chloride solution, mixed and
incubated at 22°C for 5 minutes before the addition of
15 3mg BCPB beads. RNA binding efficiency compared with a
parallel reaction with no acetyl chloride in two separate
experiments indicated that 4.2 and 5.4 times more RNA
bound to the beads when the serum had been pre-
acetylated. This enhancement of RNA binding may be the
20 result of amino-acids and carbohydrates in the serum
becoming acetylated and therefore not competing with the
2'-OH groups of the RNA for the BCPB beads.

Example 15

25 RT-PCR amplification of RNA purified from serum
100ng of BMV RNA (Promega, USA) in 1 μ l of water was added
to 1 μ l of fresh human serum and then mixed with 40 μ l of
either THF or DMSO in the presence or absence of
150mM sodium perchlorate or 0.5% SDS (final
30 concentrations) and then 3mg of BCPB beads added, mixed
and incubated at 22°C for 16min, the beads were then
washed once in 100 μ l of 70% ethanol, once in 100 μ l of
10% SDS and three times 100 μ l of water. The beads were

finally resuspended in 8 μ l of water before addition to the RT reaction.

One half (4 μ l) of the RNA-BCPB beads were added to a
5 20 μ l reverse transcription reaction containing the following final component concentrations: 200 mM Tris-HCl (pH 8.4 at 24°C), 75 mM KCl, 2.5 mM MgCl₂, 10 mM DTT, 1 mM dNTP's, 110ng of BMV-R oligonucleotide primer (GAGCCCCAGCGCACTCGGTC) (SEQ ID NO: 1) and 100 units of
10 MULV Point Mutant (Promega, USA). The reaction was allowed to proceed for 40 min at 42°C. The cDNA was then used directly in a PCR reaction as follows.

The PCR was carried out in a final volume of 25 μ l with
15 final concentrations of 15mM Tris-HCl pH 8.8, 60mM KCl, 2.5mM MgCl₂, 400 μ M each dNTP, 10 pmol of each primer BMV F (CTATCACCAAGATGTCTTCG) (SEQ ID NO: 2) and BMV R (GAGCCCCAGCGCACTCGGTC) (SEQ ID NO: 1) and 1 unit *Taq* DNA polymerase (Amersham_Pharma_{cia} Biotech, UK). 2 μ l of the
20 bead complex/cDNA reaction was added per reaction. Cycle parameters were 94°C x 10 sec, 55°C x 10 sec and 72°C x 15 sec for 30 cycles. PCR products were visualised following agarose gel electrophoresis and staining with EtBr. It was found that none of the
25 reactions in THF led to an amplification product even if sodium perchlorate or SDS had been added. However, all the DMSO reactions provided a suitable template for RT-PCR, with the addition of 150mM sodium perchlorate or 0.5mM SDS (final concentrations) increasing the amount of
30 PCR product by 4.3 and 11 times respectively. Other successful RT-PCR reactions were obtained from 100ng BMV RNA templates purified from 1 μ l of serum using 10 μ l of DMSO and 3mg BCPB beads containing 15 or 150mM sodium

perchlorate, or 2% SDS, or 400mM guanidine thiocyanate or 600mM urea. BCPB beads are therefore useful to purify RNA for RT-PCR analysis from serum.

CLAIMS:

1. A preparative method for isolating RNA comprising an oligo- or polynucleotide from a sample, which method comprises:
 - 5 (a) treating the sample with a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the RNA under conditions so that a proportion of the 2'-OH positions of the ribose rings bear a substituent; and
 - 10 (b) preparing isolated RNA therefrom by separating material containing the substituent from the sample on the basis of a property of the substituent.
- 15 2. A method according to claim 1, wherein step (a) is carried out in a reaction medium which comprises an organic solvent.
3. A method according to claim 2, wherein the organic solvent comprises an organic base.
- 20 4. A method according to claim 2 or claim 3, wherein the reactant comprises an acid anhydride, an acid chloride, a carboxylic acid or an N-acylimidazole.
- 25 5. A method according to claim 4, wherein the reaction medium further comprises an acylation catalyst.
6. A method according to any one of claims 2 to 5, wherein the reaction medium further comprises water.
- 30 7. A method according to any one of the preceding claims, wherein the RNA comprises mRNA, rRNA or viral RNA.

8. A method according to any one of the preceding claims, wherein the sample comprises a sample from a biological source.

5 9. A method according to any one of the preceding claims, wherein the sample includes DNA.

10 10. A method according to any one of the preceding claims, wherein the substituent comprises a solid phase.

10 11. A method according to claim 10, wherein the solid phase comprises benzoyl chloride polymer bound (BCPB) beads, silica particles or particles of a glass.

15 12. A method according to claim 10 or claim 11, wherein the solid phase is modified to introduce a reactive group which reactive group is capable of reacting with RNA to capture the RNA on the solid phase.

20 13. A method according to claim 12, wherein the reactive group is introduced by modifying the solid phase with a bi-functional acid halide.

25 14. A method according to any one of claims 1-9, wherein the substituent comprises a hydrophobic substituent.

15. A method according to claim 14, wherein the hydrophobic substituent comprises a substituent, OR, 30 wherein R comprises C₁-C₃₆ alkyl; C₁-C₃₆ alkenyl; C₁-C₃₆ alkynyl; C₁-C₃₆ haloalkyl; C₁-C₃₆ aminoalkyl; C₁-C₃₆ alkoxyalkyl; C₁-C₃₆ alkylthioalkyl; C₁-C₃₆ alkoxyalkoxyalkyl; C₁-C₃₆ haloalkoxyalkyl; C₁-C₃₆ aminoalkoxyalkyl; C₆-C₃₆ aryl; C₆-C₃₆ alkylaryl; C₆-C₃₆ 35 arylalkyl; C₆-C₃₆ arylalkenyl; C₁-C₃₆ alkanoyl; C₁-C₃₆

alkenoyl; C₁-C₃₆ haloalkenoyl; C₁-C₃₆ haloalkanoyl; C₂-C₃₆
haloformylalkanoyl; C₁-C₃₆ C₁-C₃₆ aminoalkanoyl; C₁-C₃₆
azidoalkanoyl; C₁-C₃₆ carboxyalkanoyl; C₁-C₃₆
carboxyalkenoyl; C₁-C₃₆ carboxyalkynoyl; C₁-C₃₆

5 alkylaminoarylalkanoyl; C₁-C₃₆ alkoxy carbonyl; C₁-C₃₆
alkenyloxy carbonyl; C₁-C₃₆ alkylsulfonyl; C₆-C₃₆
arylalkanoyl; C₆-C₃₆ arylalkenoyl; C₆-C₃₆ aryloxyalkanoyl;
C₆-C₃₆ alkylarylalkanoyl; C₆-C₃₆ haloarylalkanoyl; C₆-C₃₆
aminoarylalkanoyl; C₁-C₃₆ alkylsilanyl; C₁-C₃₆

10 trialkylsilanyl or C₁₂-C₂₈ diarylphosphano; or a
substituent R', wherein R' comprises C₁-C₃₆ alkyl; C₁-C₃₆
alkenyl; C₁-C₃₆ alkynyl; C₁-C₃₆ haloalkyl; C₁-C₃₆
aminoalkyl; halo; amino; C₁-C₃₆ alkylamino; C₆-C₃₆ aryl;
C₁-C₃₆ alkylaryl or C₁-C₃₆ arylalkyl.

15

16. A method according to claim 15, wherein the hydrophobic substituent comprises a C₄ to C₈ carbon chain or ring.

20 17. A method according to claim 16, wherein the reactant comprises butyric anhydride, pentanoic anhydride, hexanoic anhydride or benzoic anhydride.

25 18. A method according to claim 16 or claim 17, wherein the proportion of 2'-OH positions bearing the substituent is at least 10%.

30 19. A method according to claim 15, wherein the hydrophobic substituent comprises a C₈-C₁₂ carbon chain or ring.

20. A method according to claim 19, wherein the proportion of 2'-OH positions bearing the substituent is in the range 1 to 10%.

21. A method according to claim 15, wherein the hydrophobic substituent comprises a C₁₂-C₃₆ carbon chain or ring.

5

22. A method according to claim 21, wherein the proportion of 2'-OH positions bearing the substituent is up to 1%

23. A method according to any one of claims 14 to 10 22, wherein step (b) comprises contacting the treated sample from step (a) with a hydrophobic solid phase so as to bind the material containing the hydrophobic substituent and optionally washing the material bound to the solid phase.

15

24. A method according to claim 23, wherein the hydrophobic solid phase comprises hydrophobic particles.

25. A method according to claim 23 or claim 24, 20 which further comprises a step of eluting the material bound to the hydrophobic solid phase by treating with a detergent, a chaotrope or a solvent, by lowering the salt concentration or by cleaving the substituent from the 2'-OH position of the ribose rings.

25

26. A method according to any one of claims 14 to 25, wherein step (b) comprises treating the treated sample from step (a) with a lyotropic salt to aggregate the material containing the hydrophobic substituent as an RNA 30 precipitate, and isolating the precipitate.

27. A method according to claim 26, wherein the lyotropic salt comprises ammonium sulphate, an alkali

metal chloride, magnesium chloride or calcium chloride.

28. A method according to any one of claims 14 to 22, wherein step (b) comprises treating the treated sample 5 with a non-polar solvent to form a hydrophobic liquid phase which contains the material containing the hydrophobic substituent, and isolating the hydrophobic liquid phase.

10 29. A method according to claim 28, wherein the non-polar solvent comprises pentane, cyclohexane, toluene, benzene, light petroleum, xylene or hexane.

30. A kit for the preparative isolation of RNA 15 comprising an oligo- or polynucleotide from a sample, which kit comprises:
(i) a reaction system for modifying the RNA to form a modified oligo- or poly-nucleotide in which a proportion of the 2'-OH positions of the ribose rings bear a 20 substituent; and
(ii) a separation system for preparing isolated RNA by separating material containing the substituent from the sample on the basis of a property of the substituent.

25 31. A kit according to claim 30, wherein the reaction system comprises:
(a) an organic solvent; and
(b) a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the RNA in the presence 30 of the organic solvent.

32. A kit according to claim 31, wherein the organic solvent comprises an organic base.

33. A kit according to claim 31 or claim 32, wherein reactant comprises an acid anhydride, an acid chloride, a carboxylic acid or an N-acylimidazole.

5

34. A kit according to claim 33, which further comprises an acylation catalyst.

10 35. A kit according to any of claims 31 to 34, wherein the substituent comprises a solid phase.

36. A kit according to claim 35, wherein the solid phase comprises benzoyl chloride polymer bound (BCPB) beads, silica particles or particles of a glass.

15

37. A kit according to any one of claims 31 to 34, wherein the substituent comprises a hydrophobic substituent.

20 38. A kit according to claim 37, wherein the hydrophobic substituent comprises a substituent, OR, wherein R comprises C_1-C_{36} alkyl; C_1-C_{36} alkenyl; C_1-C_{36} alkynyl; C_1-C_{36} haloalkyl; C_1-C_{36} aminoalkyl; C_1-C_{36} alkoxyalkyl; C_1-C_{36} alkylthioalkyl; C_1-C_{36} alkoxyalkoxyalkyl; C_1-C_{36}

25 haloalkoxyalkyl; C_1-C_{36} aminoalkoxyalkyl; C_6-C_{36} aryl; C_6-C_{36} alkylaryl; C_6-C_{36} arylalkyl; C_6-C_{36} arylalkenyl; C_1-C_{36} alkanoyl; C_1-C_{36} alkenoyl; C_1-C_{36} haloalkenoyl; C_1-C_{36} haloalkanoyl; C_2-C_{36} haloformylalkanoyl; C_1-C_{36} C_1-C_{36} aminoalkanoyl; C_1-C_{36} azidoalkanoyl; C_1-C_{36}

30 carboxyalkanoyl; C_1-C_{36} carboxyalkenoyl; C_1-C_{36} carboxyalkynoyl; C_1-C_{36} alkylaminoarylalkanoyl; C_1-C_{36} alkoxycarbonyl; C_1-C_{36} alkenyloxycarbonyl; C_1-C_{36} alkylsulfonyl; C_6-C_{36} arylalkanoyl; C_6-C_{36} arylalkenoyl; C_6-C_{36} aryloxyalkanoyl; C_6-C_{36} alkylarylalkanoyl; C_6-C_{36}

35 haloarylalkanoyl; C_6-C_{36} aminoarylalkanoyl; C_1-C_{36}

alkylsilanyl; C₁-C₃₆ trialkylsilanyl or C₁₂-C₂₈ diarylphosphano; or a substituent R', wherein R' comprises C₁-C₃₆ alkyl; C₁-C₃₆ alkenyl; C₁-C₃₆ alkynyl; C₁-C₃₆ haloalkyl; C₁-C₃₆ aminoalkyl; halo; amino; C₁-C₃₆ 5 alkylamino; C₆-C₃₆ aryl; C₁-C₃₆ alkylaryl or C₁-C₃₆ arylalkyl.

39. A kit according to claim 38, wherein the hydrophobic substituent comprises a C₄ to C₇ carbon chain 10 or ring.

40. A kit according to claim 39, wherein the reactant comprises butyric anhydride, pentanoic anhydride, hexanoic anhydride or benzoic anhydride.

15 41. A kit according to claim 39 or claim 40, wherein the proportion of 2'-OH positions bearing the substituent is at least 10%.

20 42. A kit according to claim 37, wherein the hydrophobic substituent comprises a C₈-C₁₂ carbon chain or ring.

43. A kit according to claim 42, wherein the proportion of 2'-OH positions bearing the substituent is in the 25 range 1 to 10%.

44. A kit according to claim 37, wherein the hydrophobic substituent comprises a C₁₂-C₃₆ carbon chain or ring.

30 45. A kit according to claim 44, wherein the proportion of 2'-OH positions bearing the substituent is up to 1%

46. A kit according to any one of claims 37 to 45, wherein the separation system comprises a hydrophobic

solid phase for binding the material containing the substituent.

47. A kit according to claim 46, wherein the 5 hydrophobic solid phase comprises hydrophobic particles.

48. A kit according to claim 46 or claim 47, wherein the separation system further comprises an elution medium for eluting RNA bound to the hydrophobic solid 10 phase.

49. A kit according to any one of claims 37 to 45, wherein the separation system comprises a lyotropic salt for aggregating the material containing the 15 hydrophobic substituent.

50. A kit according to any one of claims 37 to 45, wherein the separation system comprises a non-polar solvent for forming a hydrophobic liquid phase which 20 contains the material containing the hydrophobic substituent.

51. A preparative device for isolating RNA comprising an oligo- or polynucleotide from a sample from a subject, 25 which device comprises:

(i) a means for extracting the sample from the subject;

(ii) a reaction system for modifying RNA in the sample to form a modified oligo- or poly-nucleotide in 30 which a proportion of the 2'-OH positions of the ribose rings bear a substituent; and

(iii) a separation system for preparing isolated RNA by separating material containing the substituent from the sample on the basis of a property 35 of the substituent.

52. A device according to claim 51, wherein the means for extracting the sample from the subject comprises a syringe needle.

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53. A device according to claim 51 or claim 52, wherein the substituent comprises a solid phase.

10 54. A device according to claim 53, wherein the solid phase comprises a membrane, a particle, a bead, a filter, a fibre, a gel, a strip, a matrix, a resin, a capillary or the walls of a vessel.

15 55. A device according to any of claims 51-54, wherein the sample comprises biological material.

56. A device according to claim 55, which device further comprises a filter for removing red and/or white blood cells.

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ABSTRACT

Provided is a preparative method for isolating RNA comprising an oligo- or polynucleotide from a sample, 5 which method comprises: (a) treating the sample with a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the RNA under conditions so that a proportion of the 2'-OH positions of the ribose rings bear a substituent; and (b) preparing isolated RNA 10 therefrom by separating material containing the substituent from the sample on the basis of a property of the substituent.

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ABSTRACT

Provided is a preparative method for isolating RNA comprising an oligo- or polynucleotide from a sample, which method comprises: (a) treating the sample with a reactant capable of covalently modifying the 2'-OH position of the ribose rings of the RNA under conditions so that a proportion of the 2'-OH positions of the ribose rings bear a substituent; and (b) preparing isolated RNA therefrom by separating material containing the substituent from the sample on the basis of a property of the substituent.